

## Exhibit 9

## Photodynamic therapy and diagnostic measurements of basal cell carcinomas using esterified and non-esterified $\delta$ -aminolevulinic acid

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**ABSTRACT:** Various optical techniques were used to investigate relevant parameters involved in photodynamic therapy (PDT) of human basal cell carcinomas (BCCs). The aim of the study was to compare the diagnostic and therapeutic outcome when using topically applied methyl-esterified  $\delta$ -aminolevulinic acid (ALA-ME) and  $\delta$ -aminolevulinic acid (ALA). A total of 35 pathologically verified BCCs in 14 patients were investigated. A diode laser, emitting continuous light at 633 nm, was used to induce PDT. The diagnostic measurements were performed before, during, and after PDT. Laser-induced fluorescence (LIF) was used to monitor the build-up of the ALA/ALA-ME-induced protoporphyrin IX (PpIX). The superficial tissue perfusion was measured with laser-Doppler perfusion imaging (LDPI) and the temperature of the lesion and the surrounding tissue was imaged with an IR-camera. A clear demarcation between the lesion and the normal skin was detected with LIF before the treatment for both PpIX precursors. The fluorescence measurements suggest that PpIX builds up to a higher degree and more selectively in the tumour following ALA-ME as compared to ALA. The LDPI measurements indicate a local transient restriction in blood perfusion immediately post-PDT. The measurement with the IR-camera revealed a temperature rise of about 1–2 °C during the treatment. Copyright © 2001 John Wiley & Sons, Ltd.

**KEYWORDS:** laser-Doppler perfusion imaging; laser-induced fluorescence; protoporphyrin IX;  $\delta$ -aminolevulinic acid; skin malignancies; thermal imaging

### ABBREVIATIONS

ALA,  $\delta$ -aminolevulinic acid; ALA-ME, methyl-esterified  $\delta$ -aminolevulinic acid; BCC, basal cell carcinoma; LDPI, laser-Doppler perfusion imaging; LIF, laser-induced fluorescence; PDT, photodynamic therapy; PpIX, protoporphyrin IX.

### INTRODUCTION

Photodynamic therapy (PDT) is an investigative treatment modality for malignant tumours based on the photosensitisation of tumour after application of a sensitiser, that following illumination together with oxygen results in tissue oxidation. The haem precursors  $\delta$ -aminolevulinic acid (ALA) and  $\delta$ -aminolevulinic acid methyl ester (ALA-ME)

are the agents used to sensitise the tissue in this study. Promising results with esterified ALA have been reported [1–3] although opposite data have also been presented [4]. The use of ALA relies on the ability of the tissue to synthesise the PDT-active protoporphyrin IX (PpIX) and has successfully been used in the PDT treatment of various skin lesions such as basal cell carcinoma (BCC) [5]. Excess amounts of exogenous ALA circumvent ALA synthase, the initial rate-limiting enzyme for haem synthesis. The next steps in the haem cycle are fast while the last step is relatively slow, resulting in PpIX accumulation. The subsequent irradiation of the photosensitised tissue with non-ionising radiation generates selective damage to the tumour tissue [6–8], either by direct toxicity to the tumour cells or by inducing cellular hypoxia.

Microcirculatory shut-down may be induced by direct toxicity to the endothelial and vascular smooth muscle cells or by release of vasoactive substances [8]. Endothelial cells are usually quiescent in normal tissues, but are stimulated to proliferate in the presence of tumour-derived growth factors, and may therefore respond differently to PDT. Since it has been demonstrated that endothelial cells are sensitive to PDT-induced cytotoxicity and that they

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accumulate 1.5 to 4 times [9] more PpIX when proliferating than when quiescent, these cells are important targets for PDT. In addition there is evidence that tumour cells generate more PpIX and that they therefore are more sensitive to PDT than the non-neoplastic cell lines studied [9]. For example, vascular smooth muscle cells *in vitro* were found to produce sufficient amounts of PpIX to be susceptible to PDT-induced toxicity following 4 h incubation with ALA [9].

Topical administration of ALA has so far been the most employed route of administration for PDT of BCCs. Previous studies imply that topical application is not reliable in achieving full-thickness sensitisation due to inadequate deep penetration and no inherent selectivity for malignant basal cells over normal epidermis [10]. Efforts have been made into developing prodrugs of ALA with higher lipophilicity, which is a promising approach [11].

The aim of this study was to investigate the mechanisms involved in PDT of BCCs following topical administration of ALA and ALA-ME. Laser-induced fluorescence (LIF) was used to monitor the pharmacokinetics of PpIX superficially, laser-Doppler perfusion imaging (LDPI) [12] was employed to study the superficial tissue perfusion in the treated lesion and surrounding tissue. Thermal imaging was also utilised during the laser irradiation to measure the response of the deeper vessels and to monitor temperature changes due to light absorption.

## MATERIALS AND METHODS

### Patients

Patients referred to the Departments of Dermatology and Oncology at the Lund University Hospital, Lund, Sweden, for PDT of BCCs were treated. In connection with the treatments the superficial PpIX build-up and the tissue perfusion were monitored, and the temperature rise due to the treatment irradiation was measured. A total of 35 pathologically verified lesions in 14 patients were included in the study. Table 1 gives a brief overview of all patients, the treatment parameters and the different measurements performed in each lesion. (Eleven patients were included in the LIF measurements with a total of 11 lesions treated with ALA and 19 lesions treated with ALA-ME. Nine patients and 10 lesions following ALA-ME administration were included in the LDPI study. Temperature measurements were performed on five patients and 13 lesions of which eight were treated with ALA-ME and five with ALA.) Each treated lesion was also photographed. The ambient room temperature was recorded.

The aim was to measure with all techniques on all of the selected patients but due to practical and technical reasons we were not able to fulfil this. Some lesions were excluded from the study since the measurements could not be performed according to the time schedule stated in the study design. This was particularly important for the LDPI measurements immediately post-PDT due to the onset of a local inflammatory response in the treated region. The measurements scheduled 1 h post-treatment were the ones that due to patient-related practical reasons failed the most.

### Sensitising Agent

For the PDT sensitisation of the lesions, PpIX synthesised

from topically applied ALA (5-amino-4-oxopentanoic acid, Sigma, St Louis, MO) and ALA-ME (5-amino-4-oxopentanoic acid methyl ester, Sigma, St Louis, MO) were used. The sensitizer powder (ALA or ALA-ME) was dissolved in water and mixed with Essex cream (Batch 96L03A, Shering-Plough, Portugal) to yield a 20% weight-based ALA/ALA-ME concentration, respectively. The cream was then applied onto the lesion for 6 h, covering a 1-cm margin outside the visible tumour border. To prevent the drug from being smeared off from the site of action, an occlusive dressing (Tegaderm<sup>®</sup>, 3M, UK) was used to cover the lesion. To protect the area from ambient light an ordinary dressing was applied on top.

### PDT Procedure

The PDT procedure was scheduled to start at approximately six hours after application of the sensitizer cream. During PDT the lesion and the surrounding normal skin were irradiated by light with a wavelength of 633 nm through an optical fibre. As a light source a diode laser (Ceralus PDT 635, CeramOptec, Bonn, Germany) was used to deliver the light via a 400 µm silica fibre with an attached microlens (MR4-65S-XY-B0, Rare Earth Medical Inc., W. Yarmouth, MA) at the distal end. The treated area, including the lesion and approximately a 1-cm margin of surrounding skin, was thus illuminated with a uniform light distribution. By adjusting the distance between the fibre tip and the lesion the size of the illumination spot could be regulated. A total light dose of 30–60 J cm<sup>-2</sup> was delivered to the lesions. Power densities less than 175 mW cm<sup>-2</sup> were imposed on the tissue to avoid hyperthermal effects.

### Laser-Induced Fluorescence Measurements

The LIF measurements were performed with a fibre-based optical multichannel analyser system (OMA). As an excitation source a compact nitrogen laser-pumped dye laser system (Models VSL 337 and DLM 220, respectively, Laser Science, Inc., Cambridge, MA), tuned to 405 nm with a pulse length of about 3 ns, was used. The excitation light was focused via a 50% beamsplitter and a quartz lens onto the tip of a 600 µm diameter, low-fluorescence quartz fibre. The excitation pulse energy out of the fibre was approximately 1 µJ. When the fibre tip was held in contact with the tissue, a fraction of the induced fluorescence was guided back through the same fibre, reflected by the 50% beamsplitter and spectrally dispersed in a spectrometer (Model MS 125, Oriel Corp, Stratford, CT). A gated image-intensified CCD-detector having 1024 × 128 pixels (Instaspec V ICCD, Andor Technology, Belfast, UK), cooled to 1°C, was utilised to record the fluorescence spectrum ranging from 280 to 800 nm, with a resolution of the system of approximately 3 nm. To prevent any backscattered excitation light from reaching the detector a cut-off filter (Schott GG420) was placed in front of the spectrometer. In order to obtain a high signal-to-noise ratio, the fluorescence from 20 laser pulses was added for each spectrum. The spectra were displayed on a monitor and stored for evaluation.

The fluorescence measurements were performed much according to the method described in detail previously [13]. First, the autofluorescence of the tissue was measured before application of the sensitizer, to be used as a reference for the later measurements. To investigate the kinetics of the PpIX

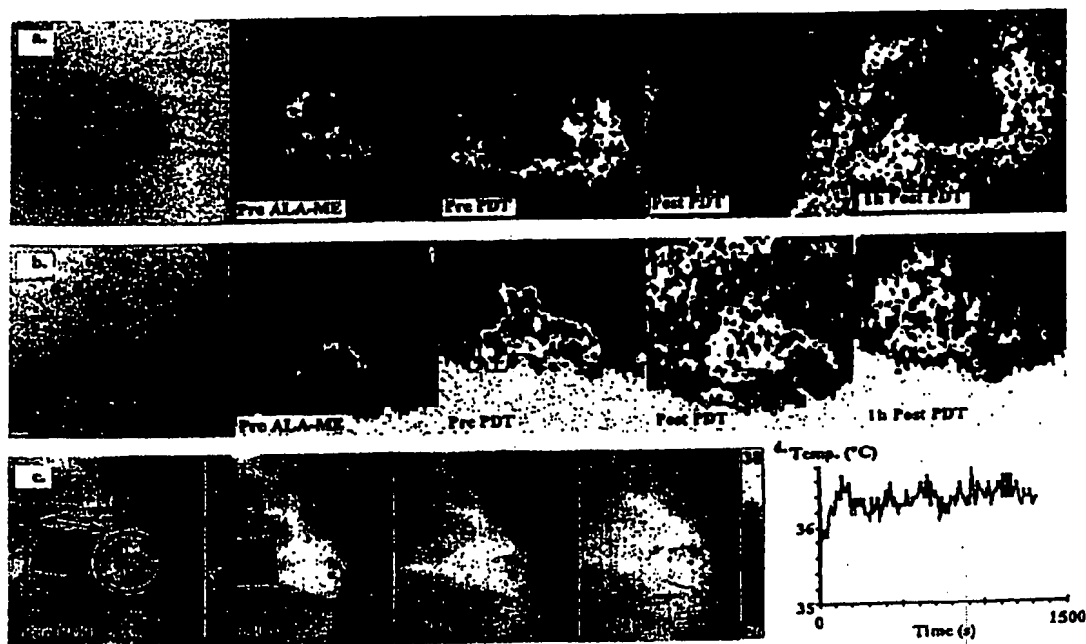


Plate 1. Laser-Doppler images recorded in (a) patient F, lesion 1, and (b) patient I, in conjunction with PDT of superficial BCC. LDPI measurements were performed before ALA-ME application, before PDT, immediately post PDT and 1 h post PDT. The range of the blood perfusion rate is from 0.00 to 10.00 (a.u.) in all images. The temperature was measured on patient I (c). The circle in the first image (c) indicates the treatment area and the area of evaluation of the maximal temperature. The temperature rise during the treatment is shown (d).

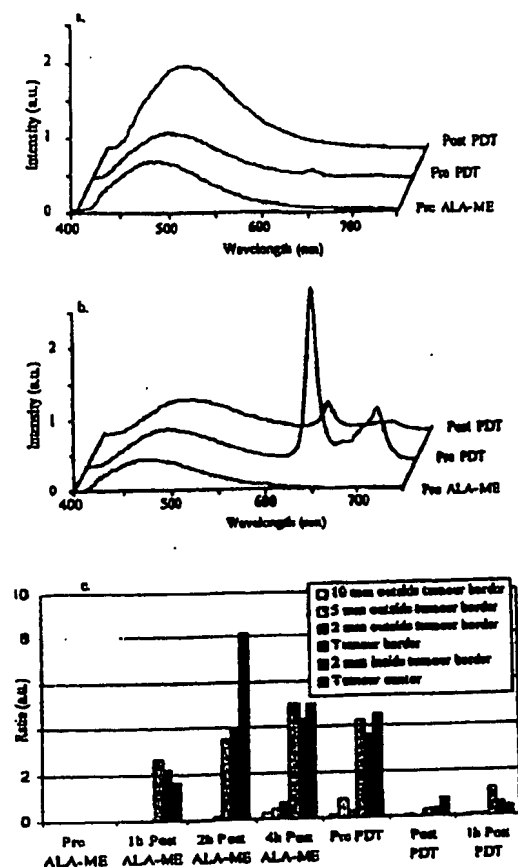


Fig. 1. Typical spectra showing build-up of the protoporphyrin peak at 635 nm for patient F, lesion 1, outside the visible tumour border (a) and at the centre of the lesion (b). It is clearly seen that the fluorescence at 635 nm in the lesion increases up until the point of the PDT procedure where it decays abruptly. This applies to the healthy tissue as well but not to the same extent. By forming the ratio between the protoporphyrin peak and the autofluorescence peak, at 480 nm, one gets a qualitative measure appropriate for comparing different types of ALA-based sensitizers (c).

1, is illustrated in Plate 1(c). The large blood vessels just outside the treated area became more visible during the initial part of the treatment. At later stages the heat was more evenly distributed in the tissue. The two darker, and thus cooler, dots in the images are two droplets of fluid on the surface of the tissue. In Plate 1(d) the maximum temperature in the illuminated area is plotted during the course of the PDT treatment for the same lesion. During the first 100 s the temperature on the surface of the tissue was raised by approximately 1–1.5 °C. After this initial increase, the temperature remains almost constant during the irradiation. The same behaviour of temperature increase and visualisation of blood vessels were found in the other lesions studied. The temperature rise after 200 s was proportional to the fluence rate ( $\Delta T(^{\circ}\text{C}) = 0.38 + 0.012 \times \text{fluence rate (mW cm}^{-2}\text{)}$ ,  $r = 0.7$ ). The average temperature rise for all patients was  $1.7 \pm 0.9^{\circ}\text{C}$  (mean  $\pm$  SD), regard-

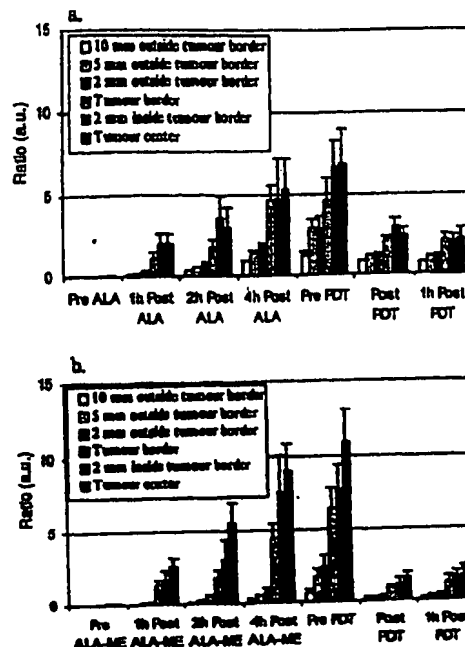


Fig. 2. In the figure the mean  $\pm$  SE of the ratio between the protoporphyrin peak, at 635 nm, and the autofluorescence, at 480 nm, for both ALA and ALA-ME can be seen. The number of BCCs included in the study was 11 and 19 respectively on 11 patients. The overall ratio in the ALA-ME-treated lesions was generally higher ( $p < 0.10$ ).

less of fluence rate, which is comparable to the temperature rise ( $1.6 \pm 0.4^{\circ}\text{C}$ ) of the healthy volunteers at a fluence rate equal to  $100 \text{ mW cm}^{-2}$  and a total fluence of  $40 \text{ J cm}^{-2}$ . A visualisation of the blood vessels was not found when illuminating the healthy tissue.

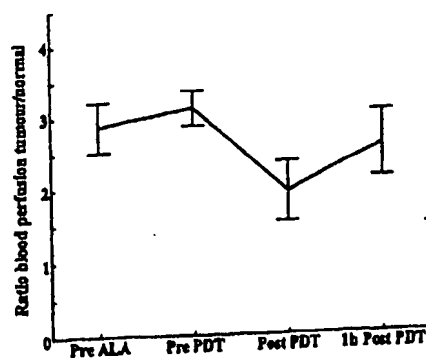


Fig. 3. Ratio of lesional blood perfusion (mean  $\pm$  SE) versus normal skin measured by LDPI before ALA-ME application, before PDT, immediately after PDT and 1 h after PDT. ALA-ME sensitisation before PDT reduces the blood perfusion at the tumour site immediately after PDT. Statistical  $t$ -test confirms decrease of the blood flow after PDT ( $p < 0.05$ ).

Table 1. Data for the patients included in this study

| Patient | Age/sex | Location     | BCC type    | Lesion size (cm) | ALA type | Fluence rate<br>(mW cm <sup>-2</sup> ) | Light doses<br>(J cm <sup>-2</sup> ) | LDPI | LIF | Temp. |   |
|---------|---------|--------------|-------------|------------------|----------|--|--------------------------------------|------|-----|-------|---|
|         |         |              |             |                  | ME       |  |                                      |      |     |       |   |
| A       | 49/M    | l. shoulder† | superficial | 4.2 × 3.2        |          | X                                      | 19                                   | 60   |     | X     | X |
|         |         | r. temple    | superficial | 1.2 × 1.2        | X        |  | 175                                  | 60   |     | X     | X |
| B       | 66/F    | chin         | superficial | 0.7 × 0.7        |          | X                                      | 113                                  | 60   | X   | X     |   |
|         |         | back         | superficial | 6.5 × 3.5        |          | X                                      | 41                                   | 60   |     | X     | X |
|         |         | back         | superficial | 0.9 × 0.7        | X        |  | 41                                   | 60   |     | X     | X |
|         |         | back         | superficial | 1.0 × 1.0        | X        |  | 113                                  | 60   |     | X     | X |
| C       | 84/F    | back         | superficial | 5.5 × 2.7        |          | X                                      | 20                                   | 60   | X   | X     |   |
|         |         | back         | superficial | 2.0 × 0.7        | X        |  | 18                                   | 60   |     | X     |   |
| D       | 44/F    | forehead     | superficial | 0.7 × 0.6        |          | X                                      | 159                                  | 48   | X   |       |   |
| E       | 80/F    | r. breast    | superficial | 4.0 × 3.5        |          | X                                      | 23                                   | 30   |     | X     |   |
| F       | 31/M    | l. clavicle  | superficial | 4.0 × 2.5        |          | X                                      | 27                                   | 30   | X   | X     |   |
|         |         | l. clavicle  | superficial | 1.3 × 1.3        |          | X                                      | 80                                   | 30   |     | X     |   |
|         | 61/F    | r. clavicle  | superficial | 1.6 × 0.6        |          | X                                      | 106                                  | 47   | X   |       |   |
|         | 42/F    | r. temple    | superficial | 0.7 × 0.7        |          | X                                      | 159                                  | 48   | X   |       |   |
|         |         | r. hip       | superficial | 0.7 × 0.6        |          | X                                      | 159                                  | 48   | X   |       |   |
| I       | 61/M    | l. shoulder  | superficial | 2.0 × 2.9        |          | X                                      | 44                                   | 60   | X   | X     | X |
| J       | 81/F    | chin         | nodular     | 0.8 × 0.8        |          | X                                      | 175                                  | 60   | X   | X     | X |
| K       | 66/M    | l. breast    | superficial | 1.3 × 1.3        | X        |  | 127                                  | 60   |     | X     | X |
|         |         | l. breast    | superficial | 1.5 × 1.5        | X        |  | 127                                  | 60   |     | X     | X |
|         |         | l. shoulder  | superficial | 1.4 × 0.7        | X        |  | 127                                  | 60   |     | X     | X |
|         |         | l. side neck | superficial | 0.5 × 0.5        |          | X                                      | 127                                  | 60   |     | X     | X |
|         |         | back         | superficial | 0.5 × 0.5        |          | X                                      | 127                                  | 60   |     | X     | X |
|         |         | back         | superficial | 1.0 × 1.5        |          | X                                      | 127                                  | 60   |     | X     | X |
| L       | 52/F    | l. clavicle  | superficial | 1.1 × 0.7        |          | X                                      | 27                                   | 30   | X   |       |   |
| M       | 50/M    | chest        | superficial | 1.5 × 0.6        | X        |  | 64                                   | 30   |     | X     |   |
|         |         | r. shoulder  | superficial | 1.5 × 1.9        | X        |  | 64                                   | 30   |     | X     |   |
|         |         | back         | superficial | 1.0 × 1.0        |          | X                                      | 64                                   | 30   |     | X     |   |
|         |         | chest        | superficial | 0.5 × 0.5        |          | X                                      | 64                                   | 30   |     | X     |   |
|         |         | r. calf      | nodular     | 1.0 × 0.8        |          | X                                      | 64                                   | 30   |     | X     |   |
|         | 36/F    | r. breast    | superficial | 0.3 × 0.3        | X        |  | 64                                   | 30   |     | X     |   |
|         |         | r. breast    | superficial | 0.3 × 0.3        | X        |  | 64                                   | 30   |     | X     |   |
|         |         | l. breast    | superficial | 0.3 × 0.3        |          | X                                      | 64                                   | 30   |     | X     |   |
|         |         | neck         | superficial | 0.3 × 0.3        |          | X                                      | 64                                   | 30   |     | X     |   |
|         |         | r. thigh     | superficial | 0.1 × 0.1        |          | X                                      | 64                                   | 30   |     | X     |   |
|         |         | back         | superficial | 0.3 × 0.4        |          | X                                      | 64                                   | 30   |     | X     |   |

† l. - left, r. - right.

build-up and accumulation, measurements were performed one, two and four hours after the application as well as immediately before the PDT treatment. The sensitiser-cream was removed during these measurements (approximately 3 min each time), and was then smeared back on the lesion area and covered again with the dressing. The last measurements were performed immediately after and one

hour after PDT to monitor the photodegradation and regeneration of PpIX following the treatment, respectively.

At each occasion, the LIF measurements were performed in scans over the surrounding skin (10, 5 and 2 mm outside the visible tumour border), on the visible border, and inside the lesion (2 mm inside the visible border and in the tumour centre), with two or more replicates at each site.

Furthermore, the peak fluorescence intensity (at approximately 580 nm) of a reference dye (10 mg Rhodamine 6G dissolved in 60 ml ethylene glycol) was recorded at each measurement occasion to compensate for fluctuations in sensitivity of the measurement equipment. The spectral response of the system was measured using a NIST traceable calibrated blackbody radiation.

#### Analysis of the Fluorescence Data

All spectra recorded at the same occasion and site were first averaged to decrease possible influence of irregularities of the lesion and surrounding tissue, although precautions were taken not to measure from areas not representative of the lesion, for instance visible blood vessels or hyperkeratotic areas. Spectral reduction of the amount of data by averaging five channels resulting in a new channel resolution of 2.5 nm and correction for the spectral-response of the system were performed in Matlab<sup>®</sup> (Mathworks Inc., Natick, MA). The autofluorescence was subtracted to evaluate the intensity of the PpIX fluorescence peak at 635 nm only. This was done by interactively fitting an exponential curve ( $I = I_0 e^{-\lambda/\lambda_0}$ ) to the fluorescence spectra in the range 530 nm to 607 nm, where there is no PpIX fluorescence. The autofluorescence in that interval was fitted by the least-square method and extrapolated to 635 nm. Small intervals in this spectral range were occasionally omitted to prevent endogenous porphyrin peaks, at about 580 nm, from affecting the fit [14]. To make a qualitative assumption of the differences between ALA and ALA-ME, the ratio between the PpIX peak (at 635 nm after subtraction of the extrapolated autofluorescence) and the autofluorescence maximum was calculated and evaluated. A Student's two-sided *t*-test was performed in order to state whether there was any significant PpIX build-up difference between ALA and ALA-ME in the centre of the lesion and in the healthy tissue, assuming the autofluorescence to be independent of the drug.

#### Laser-Doppler Perfusion Imaging Measurements

LDPI is a non-contact laser imaging technique used to monitor tissue perfusion changes in the lesions and the surrounding normal skin [12]. LDPI measurements were performed before ALA-ME application, before PDT, immediately post-PDT and 1 h post-PDT. A computer-controlled optical scanner, comprising of two mirrors was used to scan a low-power diode laser beam, step by step, in a rectangular pattern over the skin surface. The light penetrates the tissue to a depth of about 0.2 mm and a maximum of 4096 measurement sites are recorded [15]. The displayed image shows the blood perfusion in false colour coding, red corresponding to a high perfusion followed by yellow, green, light blue and finally dark blue. Absence of signal due to irregular topography of the skin is coded as grey. The ambient room temperature was kept within  $22 \pm 1^\circ\text{C}$  for all measurement procedures. The patients did not perform any exercise and relaxed at least 15 min prior to the measurements.

#### Analysis of LDPI Images

The computer software LDISOFT 1.0 (Linca Development AB, Linköping, Sweden) was used for analysis of LDPI images. The mean and standard deviations of the blood perfusion in the actual tumour region and in the nearby

normal skin were calculated for each lesion. A paired *t*-test was used to determine the statistical significance between mean ratios of lesion versus normal skin in patients before PDT and those in the same patients immediately post-PDT. A *p*-value of less than 0.05 was considered statistically significant.

#### Thermal Imaging of the Lesions

Temperature measurements using an uncooled infrared camera (AGEMA 570 Elite, Flir Systems Inc., Sweden) were performed during PDT of 13 lesions in five patients. The camera is sensitive to IR wavelengths between 7.5–13  $\mu\text{m}$ , corresponding to grey-body radiation and measures temperatures in the range of  $-20^\circ\text{C}$  to  $500^\circ\text{C}$ . The temperature resolution is  $0.1^\circ\text{C}$  at  $30^\circ\text{C}$ . During the treatment temperature images were recorded every 10 s. The emissivity of human skin was set to 0.98 [16]. A 3 cm  $\times$  3 cm blasted aluminium plate was used as a ruler in the images. For the evaluation, the maximum temperature in the area of illumination was plotted versus time. In the same way we measured the temperature rise during laser illumination without sensitiser on the shoulders of seven healthy volunteers with light skin type. Two different areas were illuminated with fluence rates of 50 and 100  $\text{mW cm}^{-2}$  respectively, for a time period of 400 s.

## RESULTS

#### Laser-Induced Fluorescence Measurements

A typical scan of a lesion can be seen in Fig. 1 where the fluorescence from patient F, lesion 1, is visualised at the different measurement times. The geometric spots chosen are 10 mm outside the tumour border (Fig. 1(a)) and the centre of the lesion (Fig. 1(b)). The two peaks at 635 nm and 710 nm, originating from PpIX, are much less prominent in the healthy skin as compared to the centre of the lesion ( $p < 0.001$ ) for both types of sensitisers. The ratio between the PpIX intensity at 635 nm and the autofluorescence at 480 nm for all measurement times can be seen in Fig. 1(c).

Figure 2 shows the mean values and standard error (SE) of the ratios for all lesions treated with ALA (Fig. 2(a)) and ALA-ME (Fig. 2(b)), respectively. As can be seen, the ratio pre-PDT at the centre of the lesions is higher with ALA-ME (mean = 10.6) than with ALA (mean = 6.8) indicating a higher accumulation of PpIX ( $p < 0.10$ ). At the same time the mean ratio is slightly, but not significantly, lower 10 mm outside the tumour border for the ALA-ME (mean = 0.64) compared to ALA (mean = 1.2).

#### Laser-Doppler Perfusion Imaging Measurements

The tissue perfusion following PDT with topical ALA-ME photosensitisation showed a decreased blood flow immediately after the treatment (Plate 1, Fig. 3). This indicates that the treatment modality of ALA-ME photosensitisation causes transient vascular constriction in the treated tissue thus reducing the oxygen supply to the tumour.

#### Temperature Measurements

A sequence of images from the treatment of a BCC sensitised with ALA-ME, located on the shoulder of patient

## DISCUSSION

To be able to optimise the photodynamic treatment of BCCs it is crucial to consider the limiting factors. The absorption of the treatment light at 633 nm is relatively low, which indicates that the treatment effects can be reached to a depth of more than 3 mm [17]. The slow penetration of ALA into the tissue, yielding an uneven distribution of PpIX has therefore been considered the most important limiting factor in achieving better ALA-PDT results in thick BCCs. The aim of the present study was to investigate how the pharmacokinetic behaviour of PpIX, as well as the tissue perfusion, were altered by replacing topically applied ALA for photodynamic treatment of BCCs with its methyl-esterified version ALA-ME. Previous studies have shown that ALA-ME penetrates better into skin than ALA [2, 18]. The esterified prodrug is enzymatically converted to ALA before it enters the haem cycle, resulting in identical synthesis of PpIX for the substances following tissue penetration [11]. The major differences between the two agents foreseen, would thus be the amount and distribution of PpIX metabolised in the tissue, due to differences in diffusion into the tissue of the two PpIX precursors.

To evaluate the degree of build-up and the selectivity of ALA-ME/ALA-induced PpIX in the superficial part of malignant skin lesions, the PpIX fluorescence was measured. Only the superficial part of the lesion was studied with this technique, since the penetration of the excitation light is limited to a few hundred micrometres [19]. The ratio between the PpIX fluorescence at 635 nm and the autofluorescence at around 480 nm was analysed in this study, making absolute intensity calibration unnecessary. A significantly higher PpIX-to-autofluorescence ratio was obtained immediately prior to the PDT treatment, six hours after sensitiser application, in lesions treated with ALA-ME as compared to those treated with ALA. Under the assumption that neither ALA nor its metabolites affect the tissue autofluorescence signal recorded, ALA-ME yields a higher PpIX concentration in the superficial part of the lesion than ALA. The data obtained also indicate that there was a higher degree of selective accumulation of PpIX using ALA-ME as compared to ALA. This was evaluated as the ratio between the PpIX-to-autofluorescence ratio in the central part of the lesion and in a region 5–10 mm outside the visible lesion tumour border.

The method used in analysing the data is very simple and straightforward. The autofluorescence signal is used as an internal intensity standard, to eliminate the need of absolute intensity calibration. This method was judged to be sufficiently sophisticated for comparing the two types of agents for PDT. The major advantage of using the ratio is that many unknown systematic errors are being cancelled: excitation intensity, distance between fibre tip and measurement site etc. A drawback is that the noise in the evaluated ratio signal in some cases originates mainly from the autofluorescence signal. Also, the formed fluorescence intensity ratio cannot be used to assess the absolute value of the PpIX concentration, since the autofluorescence is not known. The optical properties of the two detection wavelengths are also different in tissue.

Unfortunately the variations in the measured ratios between the different lesions are quite high. These variations might partly be due to spatial variations in the signal within a lesion. To reduce the effects of such spatial variations, fluorescence from several sites representative for

the area was recorded and averaged. The selection of a few points only to represent the fluorescence from the entire lesion or surrounding normal skin might not be enough. A fluorescence imaging technique may thus improve the characterisation [20, 21].

A difference in PpIX distribution following application of the two agents may also result in differences in treatment mechanisms. In this study the tissue perfusion was measured in connection with the PDT irradiation. Tissue perfusion is a very important parameter for the PDT outcome, since this treatment modality is oxygen dependent. Also, a permanent shut-down of the tissue perfusion will in itself lead to a destruction of the lesion.

The microcirculation in the treated lesion may be impaired in different ways by the treatment. Vasoconstriction might occur during the treatment due to a local release of vasoactive substances. Furthermore, blood coagulation may yield a shutdown of the local blood supply, and the capillary walls may be destroyed. Direct cytotoxicity to the microcirculation will occur only if the endothelial and vascular smooth muscle cells produce PpIX on exposure to ALA or ALA-ME. At the present time there is conflicting evidence as to whether this occurs [22, 23]. It has previously been demonstrated that systemic administration of ALA and Photofrin decreases the tissue perfusion after PDT, probably due to a high degree of necrosis of the endothelial cells causing vasoconstriction and permanent shut-down due to blood coagulation and tissue infarction [24]. Other studies show that the tissue perfusion is increased some time after PDT following topical administration of ALA [25], in a reaction similar to an inflammatory response. There is no evidence of detection of ALA in the blood stream when the agent is applied topically [26]. It seems that a critical difference in distribution following the two routes of application is the presence of PpIX in the endothelial cells of the vessels. The results presented in this study suggest a potent but transient restriction in the perfusion of the treated lesions following topical application of ALA-ME, while the perfusion in the surrounding tissue increases. This possible difference in tissue perfusion following topical application of ALA and ALA-ME needs to be further investigated. It could also be of interest to study at which point the blood restriction is induced to determine whether this might interfere with the PDT effect. One could speculate whether a difference could be due to that ALA-ME may penetrate deeper into the lesion and specifically into the endothelial cells of the vessels. It would be interesting to investigate whether ALA-ME and PpIX is present in the blood stream after topical administration and to use fluorescence microscopy of biopsies to study the distribution of PpIX following topical application of the two precursors, respectively.

The thermal imaging performed indicated that not only the superficial perfusion but also the deeper tissue perfusion increased during the treatment following ALA-ME application. The skin temperature is lightly increased during the treatment, due to the absorption of light. Tissue perfusion is expected to increase during the treatment irradiation due to the induced increase of the skin temperature [27]. The measurements also show a small increase of the temperature, well below the limit of hyperthermia, during PDT. Further, the temperature rise is not more pronounced during PDT than that of illuminating healthy tissue without sensitiser. The visualisation of the deeply lying blood vessels was not seen on the healthy volunteers, suggesting that the increased blood flow seen during PDT is not due to